Proc. Natl. Acad. Sci. USA
Vol. 76, No. 6, pp. 2853–2857, June 1979
Developmental Biology

Hemoglobin ontogeny during normal mouse fetal development

(embryonic hemoglobin/adult hemoglobin/yolk sac/fetal liver/tail-short mutation)

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Communicated by Paul A. Marks, March 15, 1979

ABSTRACT **Pure populations of large, nucleated erythrocytes derived from yolk sac blood islands were obtained during normal fetal mouse development. Embryonic hemoglobins were present in these cells early in gestation. Later in gestation, an increasing amount of adult hemoglobin was also synthesized and accumulated in this population of primitive nucleated erythrocytes, as demonstrated by both biochemical and immunocytochemical techniques.**

In the normal mouse, which has a gestational period of about 20 days, red blood cell formation occurs at around day 8 of gestation in the yolk sac blood islands. Twenty-four hours later, the primitive nucleated erythrocytes formed in the yolk sac are released into circulation where they proliferate and mature in a relatively synchronous manner. These cells persist in circulation until about day 16 of gestation. Three embryonic hemoglobins, EI ($\chi_2\gamma_2$), EII ($\alpha_2\gamma_2$), and EIII ($\alpha_2\zeta_2$), are synthesized in this population of primitive nucleated erythrocytes (1–4).

The fetal liver becomes the major site of red blood cell formation shortly after day 10 of gestation and continues until near birth. Erythroid precursor cells proliferate and differentiate within the hepatic parenchyma and mature to become small, non-nucleated definitive erythrocytes which are then released into vascular channels beginning on day 12 of gestation (5). These non-nucleated definitive erythrocytes contain only adult hemoglobin, $\alpha_2\beta_2$ (1–3).

The investigation of the hemoglobin production in the circulating primitive nucleated erythrocytes after day 11 of gestation is complicated by the presence of fetal-liver-derived definitive red blood cells. Earlier attempts to separate these cells by centrifugation were unsatisfactory (6). In this study, highly purified populations of primitive nucleated erythrocytes were obtained by the cell separation technique of velocity sedimentation at unit gravity (7). The hemoglobin composition and synthesis in purified populations of circulating primitive nucleated erythrocytes were determined from days 10 to 14 of gestation. Evidence is presented that confirms the previously published data that early in fetal mouse development, yolk-sac-derived primitive nucleated erythrocytes contain only embryonic hemoglobins (1–4). With further fetal growth and at the gestational age when adult hemoglobin synthesis begins in fetal hepatic erythroblasts, these circulating primitive erythrocytes also begin to form and accumulate adult hemoglobin in addition to embryonic hemoglobins (8).

MATERIALS AND METHODS

Animals. Adult male mutant TSJ/Le-*Ts*/+ and normal female C57BL/6J mice were obtained from the Jackson Laboratory. TSJ/Le-*Ts*/+ mice, kindly provided by E. S. Russell, came from an inbred strain maintained for more than 40 brother–sister generations with forced heterozygosis for the mutant gene tail-short (*Ts*). One or two females were caged with a male and examined for mating plugs daily. The morning on which a plug was found was designated as day 0 of gestation. From day 12 on, normal (B6TSJ)F1-+/+ fetuses could be easily distinguished from their retarded heterozygous mutant (B6TSJ)F1-*Ts*/+ littermates (9), and mutant embryos were not included in this investigation. On days 10 and 11 of gestation, however, +/+ and *Ts*/+ fetuses could not be easily distinguished and were pooled together for this study. In some immunocytochemical experiments, fetuses of (B6SJL)F1 and FL/4Re strains were used.

Cells and Tissues. On days 10–14 of gestation, pregnant mice were killed by cervical dislocation. Each conceptus was removed and placed in cold 0.1 M phosphate-buffered saline (pH 7.4). Embryos were dissected free of placentas, washed, and bled into cold phosphate-buffered saline. Erythrocytes were then washed three times in phosphate-buffered saline and blood smears were made by cytocentrifuge as described (10). Over 1000 cells in a smear were counted to determine the proportions of nucleated primitive erythrocytes and non-nucleated definitive red blood cells in each preparation.

Cell Separation. Fetal erythrocytes were separated by velocity sedimentation at unit gravity (7). Up to 2×10^7 cells were suspended in 20 ml of 0.2% bovine serum albumin (Sigma) in phosphate-buffered saline and placed in an SP-120 STAPUT chamber (Johns Glass Co., Toronto, ON, Canada) under 30 ml of phosphate-buffered saline. A 600-ml, linear gradient (1–2% bovine serum albumin in phosphate-buffered saline) was introduced under the cell band and cells were allowed to sediment for 2–3 hr at 4°C. Fractions of 30 ml were collected, and those containing primitive nucleated erythrocytes were pooled. The identity of erythrocytes present in various fractions was determined cytologically and by measurements of cell size (11).

Hemoglobin Separation. Hemolysates were prepared by lysis in over 3 vol of deionized water followed by freezing and thawing. After centrifugation, up to 20 μl of the stroma-free hemolysate was mixed with 10 μl of a solution containing 10% sucrose, 0.15% bromophenol blue, 0.25% KCN, 1.0% $K_3Fe(CN)_6$, 0.05% $NaHCO_3$ (all wt/vol), and 1.0% (vol/vol) 2-mercaptoethanol. This mixture was layered over an acrylamide running gel (pH 7.5) prepared as described (12) with the exception that *dl*-dithiothreitol was added to the polymerizing solution to a final concentration of 2.5 mM. A Tris/glycine buffer at pH 8.3 (13), with added 0.1% KCN and 0.01% dithiothreitol, was used. Electrophoresis was carried out at 2 mA/tube for 2 hr at 4°C. Hemoglobin bands were identified (3, 4, 14) and quantitated by scanning densitometry of the unstained gel at 415 nm on a Gilford 2400 spectrophotometer with linear scanning attachment. The area under each peak was integrated, and adult hemoglobin was calculated as percent of total hemoglobin

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Globin Synthesis and Separation. Washed circulating erythrocytes obtained from day 10–13 fetuses were incubated *in vitro* for 90 min with [^{3}H]leucine as described (15) before they were subjected to velocity sedimentation separation. After separation and washing, the primitive nucleated erythrocytes were hemolyzed. The hemoglobins present in the hemolysate were separated by polyacrylamide gel electrophoresis with added nonisotopic adult C57BL/6J hemolysate as marker. After electrophoresis, each hemoglobin band was isolated from the gel and the hemoglobins were eluted overnight in distilled water at 4°C. Additional adult C57BL/6J mouse hemolysate was added and globins were prepared by the cold acid/acetone method. Globin chains were separated on carboxymethyl/cellulose with 8 M urea by using a linear sodium phosphate gradient as described (2). In one set of experiments, primitive red blood cells from day 13 fetuses were incubated with [^{3}H]-leucine after cell separation followed by three washes in phosphate-buffered saline and hemolysis. The stroma-free hemolysate was then treated as before.

The proportion of the synthetic rates of adult and embryonic hemoglobins was calculated from the radioactivity incorporated into the various globin chains isolated by carboxymethylcellulose/urea column chromatography.

Immunocytochemical Studies. Antisera against adult and embryonic hemoglobins were obtained by immunization of female New Zealand white rabbits with intramuscular injections of either adult C57BL/6J hemolysate or embryonic hemoglobins isolated from day 13 or 14 fetuses, according to a schedule modified from Wood *et al.* (16). Sera with antibody titers of 1:4–1:16 were obtained, but these crossreacted with both adult and embryonic hemoglobins.

Adult and embryonic hemoglobins were purified by DEAE-cellulose anion-exchange chromatography (17) and their purities were verified by polyacrylamide gel electrophoresis. The majority of the embryonic hemoglobins thus isolated were the EII type. These purified hemoglobins were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia), which were then used for affinity purification of the rabbit antisera. Antihemoglobin antibodies were eluted from the homologous hemoglobins by incubation in 0.2 M glycine·HCl (pH 3.0).

These partially purified antibodies were further subjected to anion-exchange chromatography (18) and subsequently conjugated with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate (BBL BioQuest, Oakville, ON, Canada). Fluorescent dyes not coupled to IgGs were removed by gel filtration on Sephadex G-25. The labeled IgGs were absorbed with heterologous hemoglobins in order to insure monospecificity, which was confirmed by the Ouchterlony double-diffusion method.

Peripheral blood smears from mouse fetuses were prepared by cytocentrifugation, allowed to air-dry at room temperature, and fixed in acetone/methanol (19). These smears were stained with labeled antihemoglobin IgGs for 30 min at room temperature. After washing, these were examined in a Zeiss microscope equipped for reflected light fluorescence microscopy and transmission light phase contrast microscopy.

Table 1. Proportion of adult hemoglobin in purified nucleated yolk-sac-derived erythrocytes

Day of gestation	No. of experiments	% nucleated yolk-sac-derived erythrocytes	% adult hemoglobin
10	1	99.4	0
11	3	99.8 ± 0.05	0
12	3	99.9 ± 0.05	5.4 ± 0.4
13	5	99.4 ± 0.10	10.2 ± 0.8
14	3	98.1 ± 0.50	12.4 ± 0.6

Data are presented as mean ± SEM. The purified nucleated yolk-sac-derived erythrocytes were obtained by velocity sedimentation; at least 1000 cells were counted in each experiment. Hemoglobins were analyzed by polyacrylamide gel electrophoresis and quantitated spectrophotometrically.

RESULTS

Cell Separation. Almost all of the circulating erythrocytes on days 11 and 12 of gestation were large primitive nucleated erythrocytes. Subsequently, increasing numbers of small definitive non-nucleated erythrocytes were released from the fetal liver and, by day 14, these small erythrocytes accounted for close to 75% of the total circulating red blood cells (Fig. 1).

The mean cell volume of the primitive nucleated erythrocytes was at least 3 times that of fetal-liver-derived non-nucleated red blood cells (11). Therefore, these two fetal erythrocyte populations could be readily separated by means of velocity sedimentation at unit gravity, a cell separation method based primarily on cell volume differences (7). By this technique, populations of primitive nucleated erythrocytes with an average purity of 99.5% could be obtained at different gestational ages (Table 1). As illustrated in Fig. 2, cytological examination of these separated large cell fractions confirmed that almost all of the cells present had the morphological characteristics of the primitive nucleated erythrocytes derived from yolk sac blood island (20). Erythroblasts of fetal liver origin were

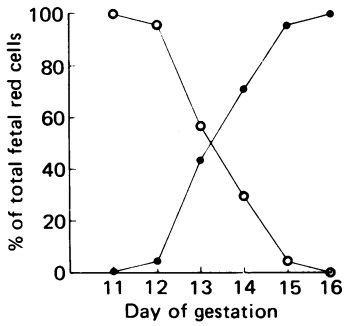

FIG. 1. Proportion of large nucleated primitive erythrocytes of yolk sac origin (○) and small non-nucleated definitive erythrocytes of fetal liver origin (●) in peripheral circulation. Embryos of the same gestation age were pooled together, washed free of maternal blood, and bled into saline. Cytocentrifuge smears of blood cells were made; over 1000 cells were counted. On each gestational day, at least three separate experiments were done.

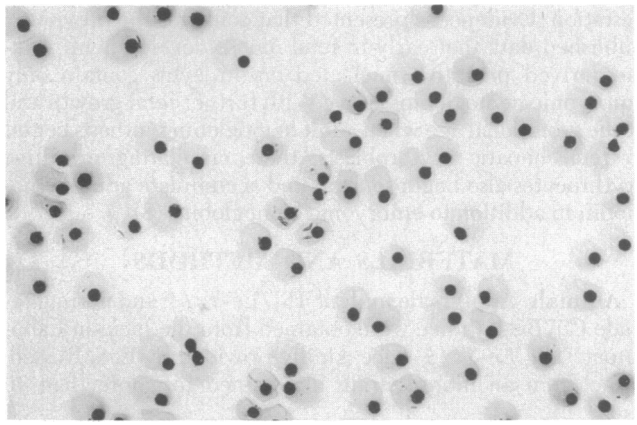

FIG. 2. Light micrograph of cytocentrifuge smear of purified large nucleated primitive erythrocytes obtained from day 14 fetuses. These cells were obtained by velocity sedimentation at unit gravity. Note the almost identical appearance of these cells with pyknotic nuclei.

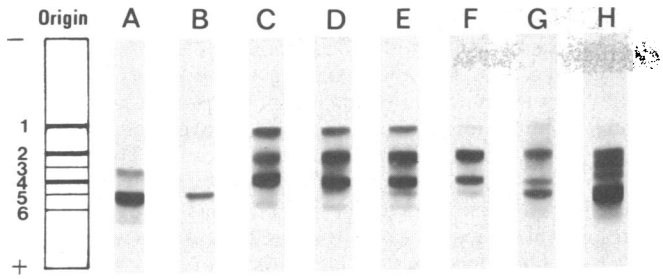

FIG. 3. Polyacrylamide gel electrophoresis of hemolysates prepared from adult and fetal blood. Hemoglobin bands are identified in the diagram according to Barker (3). Bands 1, 2, and 4 are embryonic hemoglobins EIII, EII, and EI, respectively. Band 3 is adult $Hb^{d\ minor}$; band 5 is adult $Hb^{d\ major}$ and adult Hb^{s}. Band 6 is the leading minor band (3). Gels A and B are hemolysates from peripheral blood of adult TSJ/Le-*Ts*/+ and C57BL/6J animals, respectively. Gels C–F are hemolysates prepared from pure populations of nucleated primitive erythrocytes from (B6TSJ)F1-+/+ fetuses from days 11–14 of gestation (see text): C, day 11; D, day 12; E, day 13; F, day 14. Gels G and H are hemolysates from the peripheral blood of days 14 and 15 (B6TSJ)F1-+/+ fetuses, respectively. Note the absence of band 5 in C and increasing proportion of band 5 in D–F.

morphologically distinguishable and accounted for less than 0.5% of the cells present in these large cell fractions.

Hemoglobin Studies. Adult TSJ/Le-*Ts*/+ mice had a major and minor adult hemoglobin, whereas C57BL/6J mice had only a single adult hemoglobin (Fig. 3).

Hemoglobins present in the stroma-free hemolysates prepared from purified primitive nucleated erythrocytes of yolk sac origin were examined by polyacrylamide gel electrophoresis. On day 11, four hemoglobin bands were present (gel C, Fig. 3). The fastest running band represented the "leading minor band," which has been observed in polyacrylamide gel electrophoresis of mouse hemoglobins (3). The other three bands corresponded to the three embryonic hemoglobins, EI, EII, and EIII. No adult hemoglobin was detected at this developmental stage. On subsequent gestational days, in addition to the three embryonic hemoglobin bands, an increasing amount of a hemoglobin fraction with an electrophoretic mobility identical to that of the major adult hemoglobin was present (gels D–F, Fig. 3) and by day 14 accounted for 12% of all the hemoglobins present in the primitive nucleated erythrocytes (Fig. 3 and Table 1).

Globin Synthesis. In order to confirm the identity of the putative adult hemoglobin present in yolk-sac-derived primitive erythrocytes, we analyzed globin chains by column chromatography. Circulating red blood cells obtained from day 12 and 13 fetuses were incubated *in vitro* for 90 min with [^{3}H]leucine prior to velocity sedimentation separation, hemolysis, and electrophoresis of the stroma-free hemolysate. The adult hemoglobin band was then eluted and globin chains were prepared. Globin chain separation by carboxymethylcellulose/urea column chromatography revealed that the newly synthesized radioactive globin chains were indistinguishable from the normal adult α and β globin chains (Fig. 4). The α/β synthetic ratio was 0.90 ± 0.08 in three experiments. In another experiment with cells obtained from day 13 fetuses, the primitive nucleated erythrocytes were labeled with [^{3}H]leucine after cell separation. Analysis of the radioactive globin chains in the adult hemoglobin band isolated by electrophoresis also confirmed the presence of normal adult α and β globin chains. The α/β synthetic ratio in that experiment was 0.93 (data not shown).

None of the three embryonic hemoglobins isolated from isotopically labeled day 12 and 13 fetal peripheral blood con-

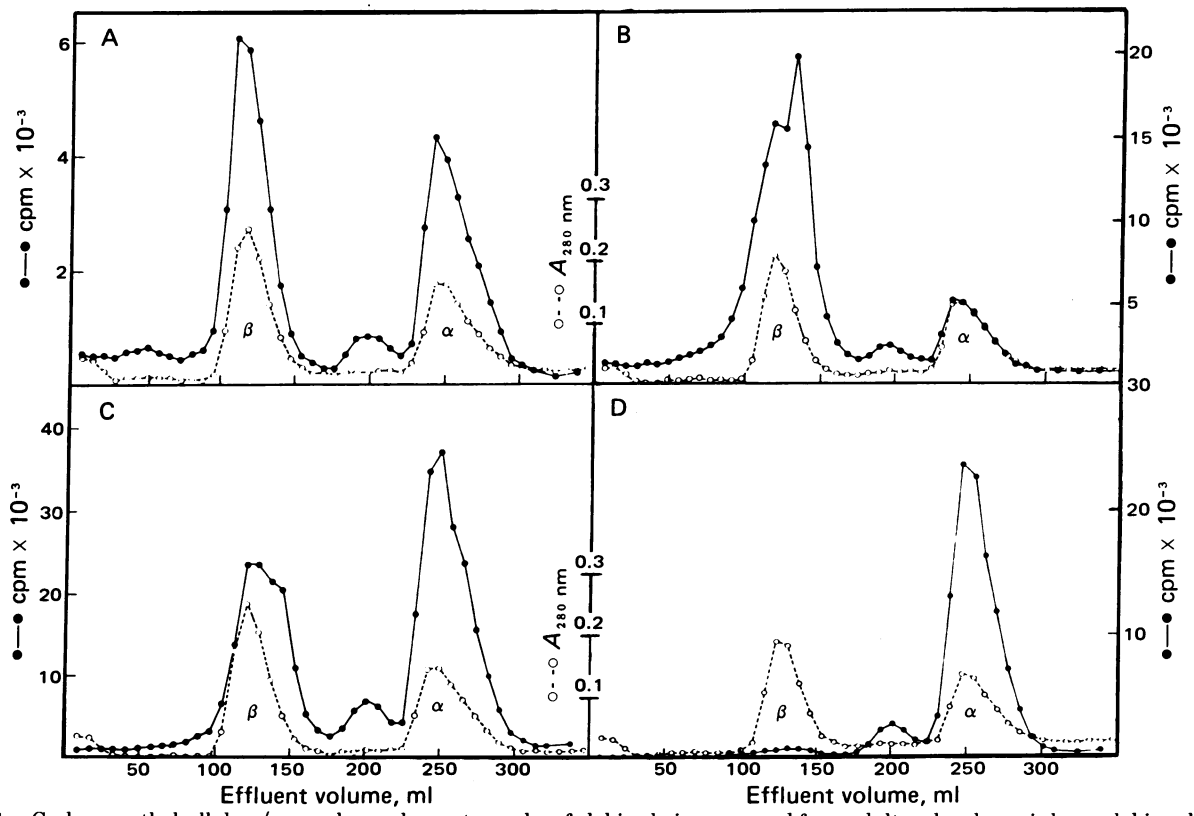

FIG. 4. Carboxymethylcellulose/urea column chromatography of globin chains prepared from adult and embryonic hemoglobins obtained by electrophoresis of hemolysate of day 13 purified primitive nucleated erythrocytes that had been pulse labeled *in vitro* with [^{3}H]leucine. Nonisotopic adult C67BL/6J hemolysate was added prior to globin preparation to provide absorbance markers for normal adult α and β globin chains. (*A*) Adult hemoglobin; (*B*) Hb EI; (*C*) Hb EII; (*D*) Hb EIII.

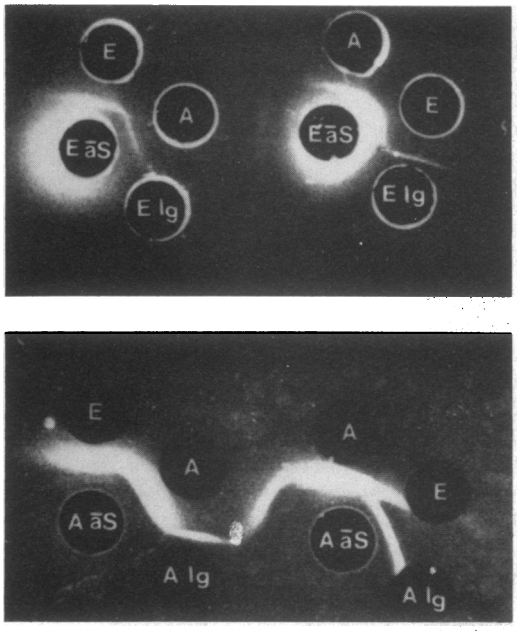

FIG. 5. Ouchterlony double-diffusion plates showing the reactivity of the antihemoglobin antisera as well as purified and fluorescence dye-labeled IgGs against purified adult hemoglobin (A) and embryonic hemoglobins (E). Eās, anti-Hb E antiserum; EIg, fluorescein-labeled purified anti-Hb E IgG; Aās, anti-Hb A antiserum; AIg, fluorescein-labeled purified anti-Hb A IgG.

tained both adult α and β globin chains (Fig. 4). Analysis of the globin chains prepared from Hb EI, composed of x_2y_2, revealed only isotopically labeled y globin chains on day 13. Hb EII, α_2y_2, contained both radioactive α and y globin chains. Hb EIII, α_2z_2, contained only radioactive α globin chains. The detection of newly formed α, β, and y globin chains in day 13 primitive nucleated erythrocytes indicated that Hb A and Hb EII were synthesized. The presence of labeled y and α globin chains in Hb EI and EIII, respectively, was probably due to the exchange of globin chains. These results are consistent with the previously published data that Hb EI and Hb EIII were not synthesized on day 13 of gestation (14).

On day 11, analysis of the globin chains prepared from the adult hemoglobin band revealed isotopically labeled normal adult α and β globin chains. However, the α/β globin synthetic ratio (0.29) was markedly lower than on day 13.

On day 10 of gestation, analysis of globin chains prepared from the adult hemoglobin band revealed the presence of radioactive embryonic x globin chains and another radioactive globin peak near, but not identical to, the adult β chains. It is likely that the isotopically labeled globin peaks represented x and y globin chains, components of Hb EI which migrated next to the adult hemoglobin band in electrophoresis. The presence of newly synthesized adult β globin chains could not be documented with confidence. On the same day of gestation, the presence of radioactive α, x, and y globin chains was demonstrated. The globin chains prepared from Hb EIII, α_2z_2, chromatographed very near to the α globin marker, but failed to resolve into two distinct peaks. However, the radioactive globin peak was skewed compared to the normal α globin chains. These results suggested that z chains were also synthesized, but they chromatographed near to the α globin chains. Taken together, these data indicated that all three embryonic hemoglobins were actively synthesized on day 10 of gestation as described (14).

Adult hemoglobin synthesis in yolk-sac-derived primitive nucleated erythrocytes could not be definitively demonstrated on day 10 of gestation. On day 11, adult hemoglobin synthesis accounted for approximately 4% of the total hemoglobin synthesis in these primitive red blood cells. The synthetic rate of adult hemoglobin, expressed as the proportion of the total hemoglobin synthesis in the primitive nucleated erythrocytes, was 9.9% on day 12 (one experiment) and 8.7% on day 13 (two experiments).

Immunocytochemical Studies. The specificities of the fluorescein- or rhodamine-labeled antihemoglobin IgGs were confirmed by the Ouchterlony double-diffusion method (Fig. 5). Moreover, absorption of IgGs with homologous antigens just prior to erythroid cell staining always eliminated fluorescence staining whereas absorption with the heterologous antigens usually had little effect upon the fluorescence staining.

As illustrated in Fig. 6, all primitive nucleated erythrocytes on day 9 of gestation were stained with anti-Hb E antibodies, but not with anti-Hb A antibodies. On the other hand, all primitive nucleated erythrocytes on day 12 were stained with both anti-Hb E and anti-Hb A antibodies, indicating the presence of both embryonic and adult hemoglobins within these primitive nucleated erythrocytes. The non-nucleated erythrocytes of fetal liver or adult mouse origin were stained only with anti-Hb A antibodies and not with anti-Hb E antibodies. Primitive nucleated erythrocytes on day 13 of gestation also stained positively with both anti-Hb E and anti-Hb A antibodies.

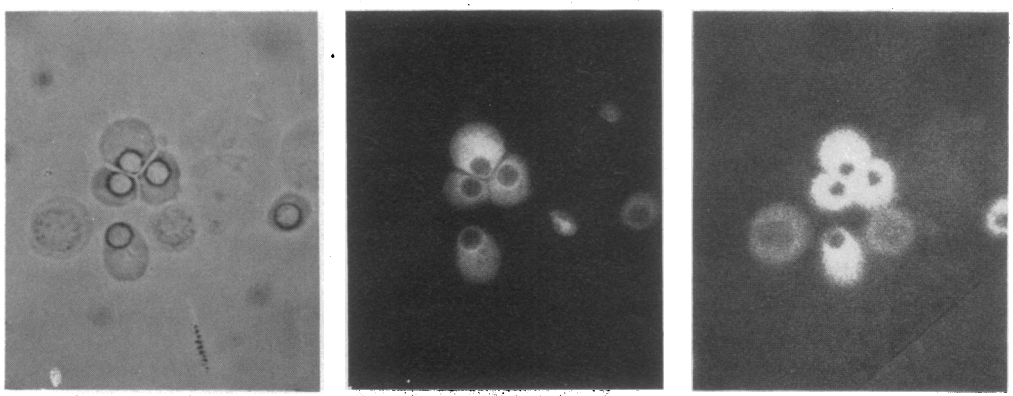

FIG. 6. Immunocytochemical studies of circulating fetal erythrocytes, double labeled with anti-Hb A and anti-Hb E antibodies. (*Left*) Phase contrast photomicrograph showing two day 9 primitive erythrocytes with high ratio of nucleus to cytoplasm, five day 12 primitive erythrocytes with small pyknotic nuclei, and two adult erythrocytes, small and not well visualized in this picture. (*Center*) Immunofluorescence of rhodamine-conjugated specific anti-Hb A. Note that all day 12 primitive erythrocytes and two adult erythrocytes are stained. (*Right*) Immunofluorescence of fluorescein-conjugated specific anti-Hb E. Note that all primitive erythrocytes of days 9 and 12 of gestation, but not adult erythrocytes, are stained.

DISCUSSION

This investigation confirms the previously published observation that early in gestation, primitive nucleated erythrocytes derived from mouse yolk sac blood islands contain only embryonic hemoglobins (1–4). The results also indicate that adult hemoglobin is synthesized in these circulating and maturing primitive nucleated erythrocytes later, at a gestational age when adult hemoglobin synthesis is also initiated in erythroid precursor cells present within the fetal livers. The findings of this study are consistent with the observation that fetal peripheral blood on day 12 of gestation contained about 10% of adult hemoglobin even when there were less than 5% of non-nucleated erythrocytes present (1, 21). The present studies do not rule out the possibility that adult hemoglobin is produced in extremely low quantities in primitive nucleated erythrocytes prior to day 11 of gestation.

The presence of adult hemoglobin in all of the circulating primitive nucleated erythrocytes on days 12 and 13 of gestation was clearly demonstrated by the immunocytochemical technique with specific antibodies against embryonic and adult hemoglobins. Moreover, the finding of adult hemoglobin in the highly purified populations of large primitive nucleated erythrocytes could not be due to contamination by small non-nucleated red blood cells of fetal liver origin for two reasons. First, the hemoglobin content in each fetal-liver-derived non-nucleated erythrocyte amounted to less than 50% of that of the large primitive nucleated cell (14, 22). Therefore, the contribution of adult hemoglobin in the hemolysate by the contamination of the non-nucleated erythrocytes (1–2%) should be negligible. Second, the radioactive labeling experiments of the primitive cells obtained before and after cell separation clearly demonstrated the *de novo* synthesis of adult hemoglobin in these primitive erythrocytes during days 11–13 of gestation. The slightly lower α/β synthetic ratio was likely to be due to the presence in the primitive erythrocytes of a pool of unlabeled α globin chains which were also the components of embryonic hemoglobins EII and EIII (2, 4).

During early chicken embryogenesis, only early hemoglobins, but not late hemoglobins, were reported to be present in primitive erythrocytes isolated by velocity sedimentation (23). In another study, both early and late hemoglobins were produced in the same population of chicken red blood cells cultured *in vitro* (24). The apparent difference between the present findings in fetal mice and those studied in chickens (23) may represent species differences, which have been reported in metamorphosing amphibians (25, 26).

On day 11 of gestation, adult hemoglobin was not detected by electrophoresis of hemolysates prepared from purified primitive nucleated erythrocytes. However, at the same gestational age, synthetic studies by pulse labeling *in vitro* with [^{3}H]leucine demonstrated that normal adult α and β globin chains were produced in these nucleated red blood cells, indicating that adult hemoglobin synthesis had begun. The synthetic rate of adult hemoglobin, expressed as the proportion of the total hemoglobin synthesis in these primitive nucleated erythrocytes, increased to about 10% on day 12 of gestation. The amount of adult hemoglobin present in these cells, as determined by electrophoresis, was 5% on day 12 and increased to slightly more than 10% on day 14 of gestation.

The gradual appearance of adult hemoglobin in yolk-sac-derived primitive nucleated erythrocytes may be the result of an intrinsic developmental program of these embryonic red blood cells. Alternatively, humoral factors may be responsible for the onset of adult hemoglobin synthesis during early mammalian embryogenesis. We have recently shown that adult hemoglobin is present in the primitive nucleated erythrocytes of the developmentally retarded mutant *Ts*/+ fetal mice at about the same gestational age as their normal littermates (9, 21). These observations support the hypothesis that circulating substances may have an important role in hemoglobin ontogeny during early mammalian fetal development.

We thank Dr. E. S. Russell for stimulating discussions and for providing the mutant *Ts*/+ mice from the Jackson Laboratory (supported by Grant CA-01074 from the National Cancer Institute) and Dr. G. D. Sweeney for introducing us to the cell separation technique. This investigation was supported by a research grant (MT-5004) from the Medical Research Council of Canada.

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